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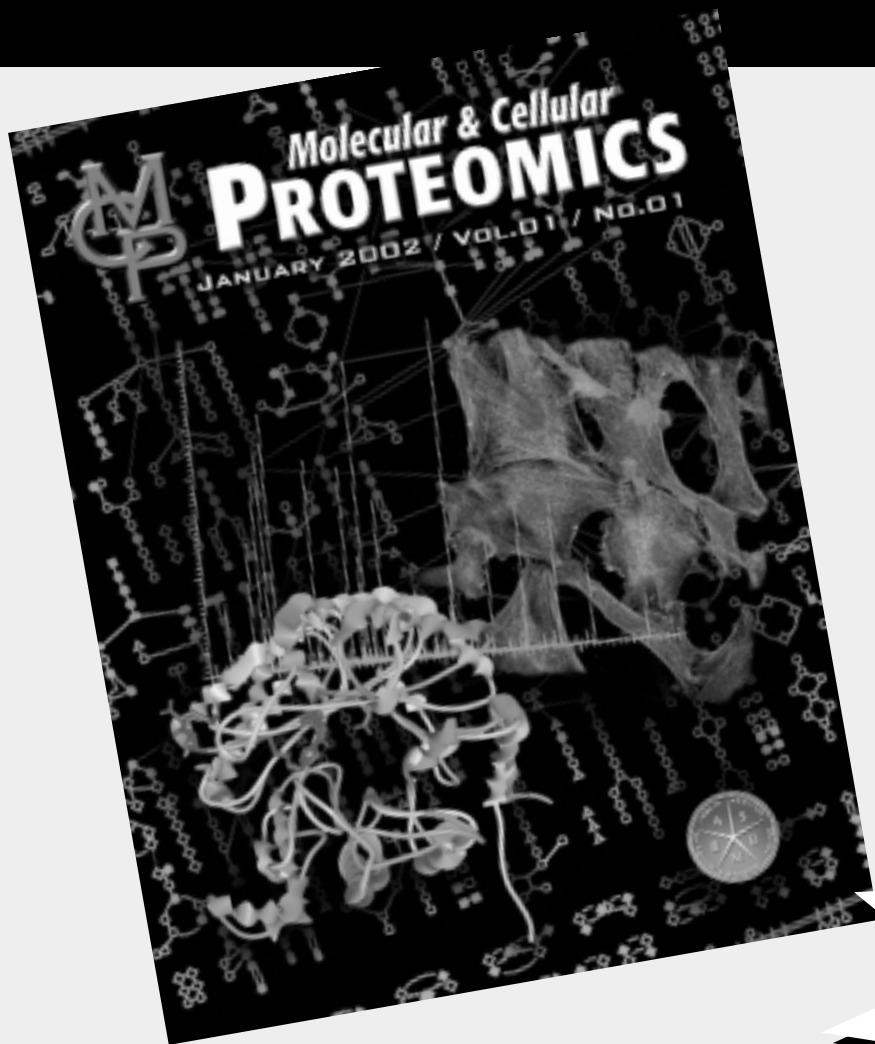
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TIN2 Mediates Functions of TRF2 at Human Telomeres*

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AQ: A

Telomeres are protective structures at chromosome ends and are crucial for genomic stability. Mammalian TRF1 and TRF2 bind the double-stranded telomeric repeat sequence and in turn are bound by TIN2, TANK1, TANK2, and hRAP1. TRF1 is a negative regulator of telomere length in telomerase-positive cells, whereas TRF2 is important for telomere capping. TIN2 was identified as a TRF1-interacting protein that mediates TRF1 function. We show here that TIN2 also interacts with TRF2 *in vitro* and in yeast and mammalian cells. TIN2 mutants defective in binding of TRF1 or TRF2 induce a DNA damage response and destabilize TRF1 and TRF2 at telomeres in human cells. Our findings suggest that the functions of TRF1 and TRF2 are linked by TIN2.

AQ: B

Telomeres are the DNA protein structures that cap the ends of linear chromosomes thereby protecting them from degradation and fusion by cellular DNA repair systems (1, 2). In mammals, telomeres consist of several kilobase pairs of double-stranded DNA that have the repeat sequence TTAGGG followed by 100–150 nucleotides of a single-stranded TTAGGG 3'-overhang. The telomeric DNA tract is thought to loop back on itself, thereby protecting the overhang, in a structure termed the t-loop (3). This protected or capped telomeric structure can be lost or disrupted by DNA damage, malfunctions in telomere-associated proteins, or as a consequence of the gradual attrition of telomeric DNA that occurs when cells proliferate in the absence of telomerase. Dysfunctional telomeres activate a DNA damage response, resulting in the binding of DNA damage response proteins to the chromosome end and induction of apoptosis or cellular senescence (4–7).

Telomere-associated proteins are crucial for forming and maintaining the protective telomeric structure *in vivo*. Some telomere-associated proteins bind directly to telomeric DNA, whereas others associate with telomeres via protein-protein interactions. Among the direct telomere-binding factors is a family of Myb domain-containing proteins that specifically bind double-stranded telomeric DNA. These proteins include Rap1p in *Saccharomyces cerevisiae* (8), Taz1 in *Schizosaccharomyces*

pombe (9), and TRF1 and TRF2 in mammals (10–12). TRF1 reduces telomere length, whereas a dominant-negative TRF1 mutant increases telomere length when overexpressed in telomerase-positive human cells (13). These results suggest that TRF1 controls the extent to which telomerase can access and hence elongate the telomeres, presumably by modulating the telomeric structure. TRF2 is essential for t-loop formation *in vitro* (14) and telomere integrity *in vivo* (3). It has been proposed that t-loop formation by TRF2 provides a mechanism to sequester the telomeric 3'-overhang, thereby preventing its degradation and/or fusion to another telomere by DNA repair processes. Disruption of TRF2 function induces ATM/p53-dependent apoptosis in human tumor cells (4) and premature senescence in normal human and mouse cells (5).

AQ: C

Several TRF1- and TRF2-interacting factors have been identified. TRF1 binds TIN2 (15), which is thought to mediate the effects of TRF1 on telomere length control and structure (16). TRF1 also exists in a complex with hPOT1 (17), the human ortholog of *S. pombe* Pot1, which binds the telomeric single-stranded 3'-overhang (18) and controls telomere length in telomerase-positive cells (17, 19). Finally, TRF1 binds TANK1 (20) and TANK2 (21), which are poly(ADP ribosyl)ases that are thought to inactivate TRF1 (22). By contrast, TRF2 interacts with hRAP1 (23), the human ortholog of scRap1p, which regulates telomere length in *S. cerevisiae* (24). TRF2 also interacts with proteins that participate in DNA repair, including RAD50 (25), Ku (26), and ERCC1 (27).

AQ: D

TIN2 interacts with TRF1 via a domain within the TRF1 homodimerization region (15), and there is no overlap between this region and the TRF1 region that binds TANK1 and TANK2 (20, 21). Consistent with being a TRF1-interacting protein, TIN2 also exists in a TRF1-hPOT complex (17). Overexpression of wild-type TIN2 slightly shortens telomeres, whereas a TIN2 mutant that binds TRF1 but lacks an N-terminal domain elongates telomeres, but both wild type and mutant function in a telomerase-dependent fashion (15, 28). In electrophoretic mobility shift assays, TIN2 is shown to form an unusually large complex with TRF1 and a telomeric DNA probe (15). These results and additional biochemical experiments (16) suggest that TIN2 mediates the telomere length control activity of TRF1 by modulating the telomeric structure.

AQ: E

AQ: F

In yeast, a single direct telomere-binding protein (Rap1p in *S. cerevisiae* and Taz1 in *S. pombe*) is the principal mediator of both telomere length and capping (9, 30), suggesting that these processes are coordinated. In mammals, however, there are two direct telomere-binding proteins, TRF1 and TRF2, which do not interact. Nonetheless, perturbations in either TRF1 or TRF2 or their associated proteins, hPOT1, hRAP1, or TIN2, influence both telomere length and capping (13, 15, 17, 23, 31), suggesting that the activities of TRF1 and TRF2 are coordinated. In addition, TRF1-deficient mouse cells show a reduction of TRF2 and TIN2 at telomeres (32), suggesting that the

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presence of TRF1 and TRF2 at telomeres may be coordinated. Here we report that TIN2 also binds TRF2 and mediates its end-capping function. We show that TIN2 interacts with TRF1 and TRF2 via distinct domains and forms complexes containing TRF1- and TRF2-interacting proteins. Moreover, TIN2 mutants elicit a DNA damage response, suggesting that TIN2 complexes are important for telomere capping in addition to telomere length control.

EXPERIMENTAL PROCEDURES

Cell Culture and Characterization—We cultured HT1080 and BJ cells as described previously (28, 33).

FLAG Affinity Purification of TIN2 Complexes—We used PCR to add a N-terminal FLAG-epitope tag to TIN2, cloned the cDNA into the retroviral vector pLXSN, and infected and selected HT1080 cells as described previously (15). Cells (6×10^6) on 150-mm culture plates were washed with phosphate-buffered saline, and then 1 ml of RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 10% glycerol, protease inhibitor tablet (Roche Applied Science)) was added to each plate. After incubation on ice for 30 min, cells were collected by scraping and centrifugation at 4 °C, and the supernatant (cell lysate) was recovered. We incubated 10 ml of supernatant with 200 μ l of anti-FLAG M2 affinity gel (Sigma) at 4 °C for 4 h. We washed the gel with RIPA buffer, released bound complexes by adding 300 μ l of 3 \times FLAG peptides (150 ng/ μ l in RIPA buffer) at 4 °C for 30 min, and collected the supernatant by centrifugation. We repeated this procedure three times as recommended by the supplier.

Immunoprecipitation and Western Analyses—We incubated cell lysates (300 μ l) in RIPA buffer with 2 μ g of anti-HA antibody (Roche Applied Science) or 10 μ g of FLAG M2 antibody (Sigma) for 2 h at 4 °C and added 50 μ l of a 50% protein A-Sepharose slurry (Amersham Biosciences) for 2 h at 4 °C. We washed the immune complexes with RIPA buffer and analyzed proteins by Western blotting as described previously (15, 34). Primary antibodies were mouse monoclonals (Imgenex) anti-TRF2, anti-TIN2, or anti-hRAP1, rabbit polyclonal anti-HA-TRF2 or anti-TRF2 or monoclonal anti-HA (Santa Cruz Biotechnology), polyclonal anti-TANK (which detects both TANK1 and TANK2) (21), polyclonal anti-TIN2 (15), and polyclonal TRF1 raised against full-length TRF1.

Yeast Two-hybrid Assays—We cloned TIN2, TIN2 mutants, TRF2, and TRF1 cDNAs into the yeast two-hybrid vectors pGBT-9, pGAD-10, pTGB-2, or pDAG-2, and vector pairs were transformed into yeast and cultured on a non-selective (TL, *-Trp, -Leu*) or selective (HTL, *-His, -Trp, -Leu*) medium with or without 3-aminotriazole as described previously (15, 16).

Immunostaining—We immunostained cells as described previously (15, 21). Briefly, we cultured cells on slide-chambers; cells were then fixed with 4% formalin, permeabilized with 0.5% Triton X-100, and stained with mouse anti-TRF2 (Imgenex), polyclonal anti-TRF1, polyclonal anti-TIN2 (15), monoclonal anti-Myc (Roche Applied Science), polyclonal anti-53BP1 (ABCcam), monoclonal anti- γ -H2AX (Upstate Biotechnology), or 10% goat serum as a control (Vector). After washing, we stained with secondary antibodies conjugated to Texas Red or fluorescein isothiocyanate (Molecular Probes) and counterstained the nuclei with DAPI. Telomeres were visualized by *in situ* hybridization using a telomeric protein nucleic acid probe as described previously (35). Where indicated, images were merged using PhotoShop (Adobe).

RESULTS

TIN2 Complexes Contain TRF2 and TRF2-associated Proteins—TIN2 interacts with TRF1 (15), with which it forms stable complexes with TRF1 on telomeric DNA (16). To better understand its function, we expressed FLAG-tagged TIN2 and/or HA-tagged TRF1 in human HT1080 fibrosarcoma cells to isolate TIN2 complexes by FLAG affinity purification. We incubated cell lysates with immobilized anti-FLAG antibody, eluted the bound proteins using FLAG-peptide, and analyzed the eluate by Western blotting.

TRF1 associates with the nuclear matrix and cannot be isolated in soluble form without harsh conditions (not shown) (36). We therefore overexpressed the tagged proteins and determined their interactions with soluble cellular proteins. In cells that overexpressed only FLAG-TIN2, the anti-FLAG antibody precipitated TRF2 and the TRF2-associated protein

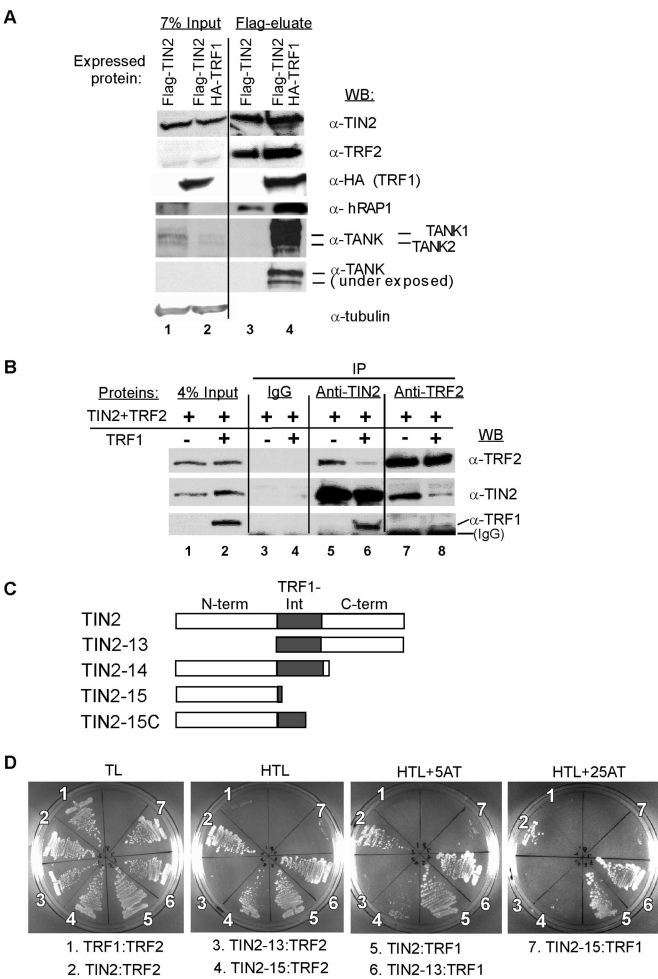


FIG. 1. TIN2 interacts with TRF2. **A**, affinity purification of TIN2 complexes is shown. We prepared lysates from HT1080 cells that stably expressed FLAG-TIN2 (lanes 1 and 3) or both FLAG-TIN2 and HA-TRF1 (lanes 2 and 4), isolated TIN2 complexes using immobilized FLAG-antibody, and analyzed the lysates (7% Input) and released complexes (FLAG-eluate) for the indicated proteins by Western blotting (WB). Two exposures are shown for TANK1 and -2, which were barely detectable in the lysates but highly enriched in the eluate. **B**, purified TIN2 interacted with purified TRF1 and TRF2 *in vitro*. Purified His₆-TIN2 (16) and His₆-TRF2 (37) in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of purified His₆-TRF1 (2 μ g each) were incubated and precipitated by control IgG (lanes 3 and 4), anti-TIN2, or anti-TRF2 antibodies. The protein mixture (4% Input) and precipitates were analyzed by Western blotting for TRF1, TRF2, and TIN2. **C**, TIN2 deletion mutants used in this study are shown. Wild-type TIN2 (aa 1–354) is depicted showing N-terminal (N-term), TRF1-interaction (TRF1-Int) and C-terminal (C-term) domains. Deletion mutants TIN2-13 (aa 196–354), TIN2-14 (aa 1–284), TIN2-15 (aa 1–209), and TIN2-15C (aa 1–257) are shown in comparison. **D**, TIN2 and TIN2-15 but not TIN2-13 interact with TRF2 in yeast. We fused TIN2, TIN2-13, TIN2-15, TRF1, or TRF2 to GAL4 functional domains in yeast two-hybrid vectors as described previously (15, 16). Proteins to the left of the column were fused to the GAL4 DNA-binding domain; proteins to the right were fused to the GAL4 transactivation domain. We transformed the indicated vector pairs into yeast and cultured and streaked the transformants on plates containing non-selective (TL) or selective (HTL) medium without or with 5 or 25 mM 3-aminotriazole (AT).

hRAP1 (Fig. 1A). The antibody did not precipitate TRF1 or the TRF1-associated proteins TANK1 and TANK2, consistent with the presence of TRF1 in the insoluble nuclear matrix fraction. However, in cells that overexpressed both FLAG-TIN2 and HA-TRF1, anti-FLAG precipitated TRF1 and the TRF1-associated proteins TANK1 and TANK2, as well as TRF2 and hRAP1 (Fig. 1A). Addition of ethidium bromide to the cell lysates did not alter these co-immunoprecipitations (not shown), indicat-

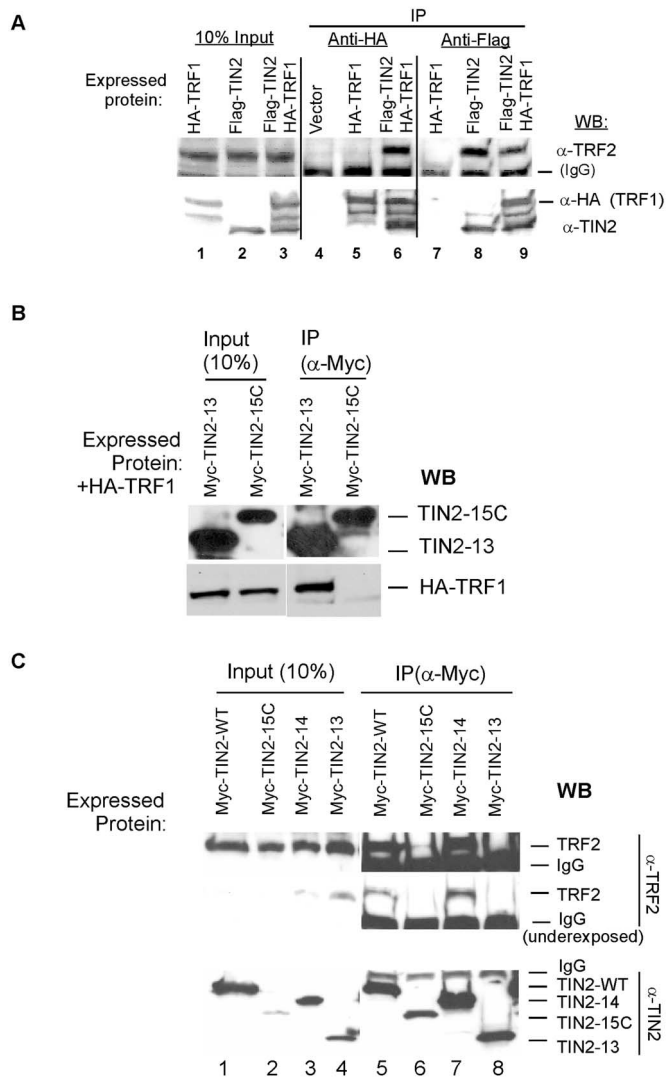


FIG. 2. Wild-type TIN2 forms a complex with TRF1 and TRF2 *in vivo*. A, interactions of TIN2 in cells are shown. Lysates from HT1080 cells stably expressing HA-TRF1, FLAG-TIN2, or both HA-TRF1 and FLAG-TIN2 were immunoprecipitated (IP) by monoclonal anti-FLAG or anti-HA antibodies. The lysates (10% Input) and immune precipitates were analyzed for TIN2, TRF1 (anti-HA), and TRF2 by Western blotting (WB). B, interactions of TIN2-15C and TIN2-13 are shown. HT1080 cells expressing HA-TRF1 were stably infected with LXSN retroviruses expressing Myc-tagged TIN2-15C or Myc-TIN2-13. Lysates were prepared and precipitated using an anti-Myc-antibody or anti-HA-antibody (the latter is not shown). Unprecipitated lysates (Input (10%)) and the immune precipitates were analyzed for TRF1 (anti-HA) and TIN2 by Western blotting. C, interactions of TIN2-13, TIN2-14, and TIN2-15C are shown. HT1080 cells were infected with LXSN retroviruses expressing Myc-tagged wild-type TIN2 or Myc-TIN2-13, TIN2-14, or TIN2-15C. Lysates were prepared and precipitated using an anti-Myc-antibody. Unprecipitated lysates (Input (10%)) and the immune precipitates were analyzed for TRF2 and TIN2 by Western blotting.

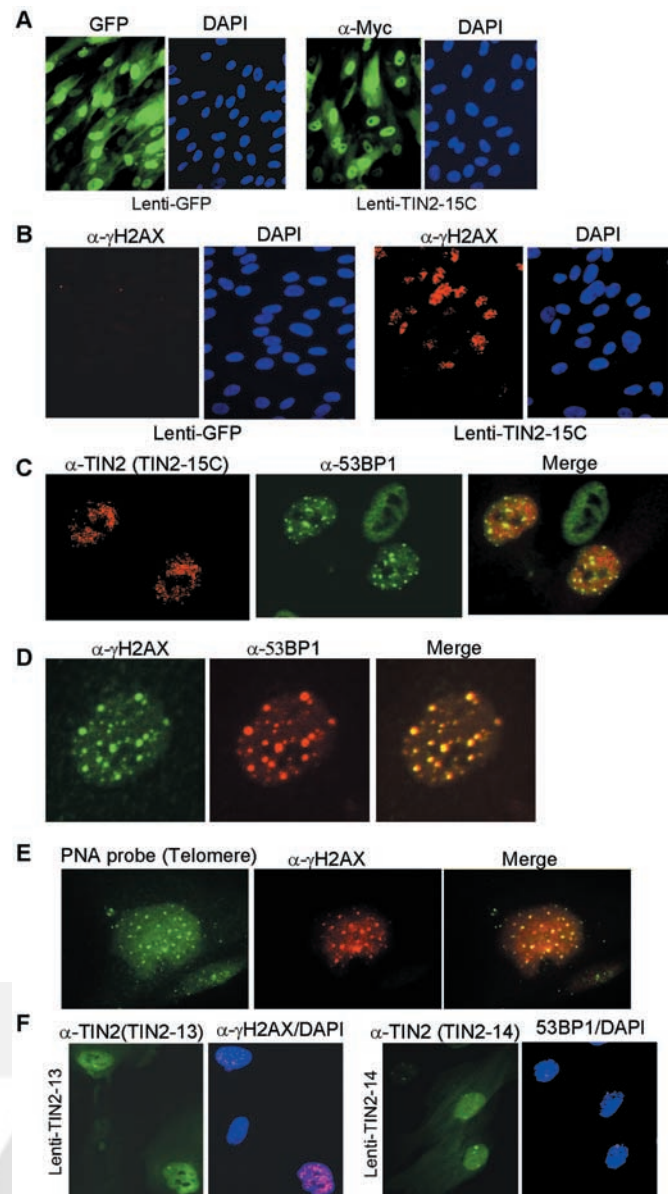


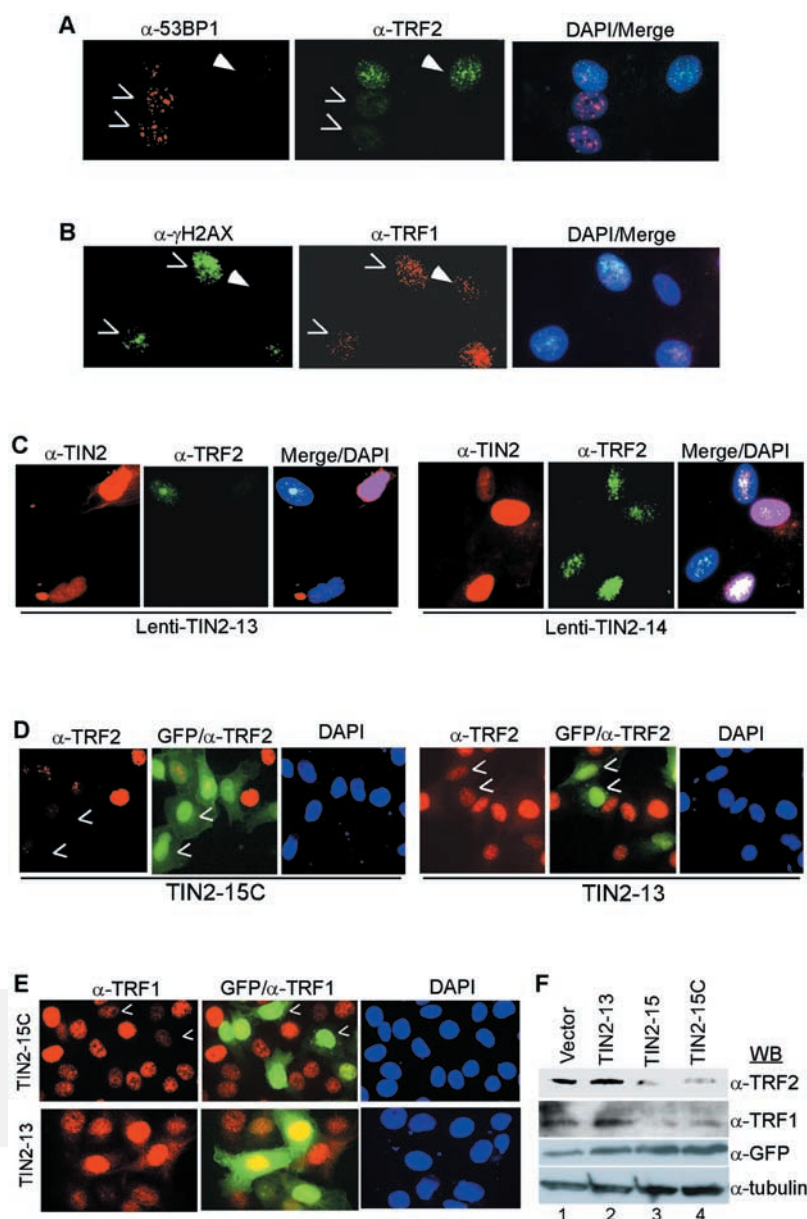
FIG. 3. Effect of TIN2 mutants on the telomeric damage response. A, lentiviral infection efficiency is shown. BJ cells were infected with lenti-GFP or lenti-Myc-TIN2-15C and monitored 48 h later for GFP fluorescence or TIN2-15C expression using Myc antibodies and immunostaining. Nuclei were stained with DAPI (blue). B and C, DNA damage response is shown. Early passage normal human fibroblasts (BJ cells) were infected with lentiviruses expressing GFP (control) or TIN2-15C (Myc-tagged) and immunostained 48 h later for γ-H2AX (b), 53BP1 (c), or TIN2-15C (anti-Myc) (c) foci. The TIN2-15C and 53BP1 images were merged (Merge). D, TIN2-15C-induced γ-H2AX and 53BP1 foci coincide. BJ cells infected with lenti-TIN2-15C were co-stained for γ-H2AX and 53BP1, and the images were merged (Merge). E, telomeric damage response is shown. BJ cells infected with lenti-TIN2-15C were co-stained for telomeres using fluorescence *in situ* hybridization and a protein nucleic acid probe, and γ-H2AX foci were co-stained using fluorescence immunostaining and a monoclonal antibody. F, TIN2-13 but not TIN2-14 induces damage foci. BJ cells were infected with lenti-TIN2-13 or lenti-TIN2-14 and stained 48 h later for expression of the mutant proteins (α-TIN2) and γ-H2AX or 53BP1 foci. The nuclei were stained with DAPI (blue) and merged with the γ-H2AX or 53BP1 images.

ing that they are not mediated by DNA. Together these results indicate that TIN2 may interact with TRF2 in addition to its known interaction with TRF1.

TIN2 Binds TRF2—To determine whether TIN2 binds TRF2 directly, we used immunoprecipitation and Western blotting to analyze the interactions of previously characterized purified proteins (Fig. 1B) (16, 37). Anti-TIN2 and anti-TRF2 specifically precipitated both TIN2 and TRF2 from TIN2-TRF2 mixtures (Fig. 1B, lanes 5 and 7), indicating a direct interaction. Interestingly, the interaction was diminished by TRF1 (Fig. 1B, compare lanes 5 and 6 with 7 and 8), and relatively little

TRF1-TIN2-TRF2 complex was detected *in vitro*. Thus, TIN2 formed primarily TIN2-TRF1 or TIN2-TRF2 complexes *in vitro*. To better understand the interactions and functions of TIN2, we created several vectors to express full-length or truncated TIN2 proteins, designated TIN2-13, TIN2-14, TIN2-15,

FIG. 4. Effects of TIN2 mutants on TRF2 and TRF1 localization and stability. A and B, TIN2-15C reduces TRF2 immunostaining at damage-induced foci. BJ cells infected with lenti-TIN2-15C were co-stained for 53BP1 and TRF2 (A) or γ -H2AX and TRF1 (B). The nuclei were counterstained with DAPI (blue), and the images were merged (DAPI/Merge). In A, solid arrowheads show 53BP1-negative (uninfected, undamaged) cell with TRF2 staining; open arrowheads show 53BP1-positive (infected, damaged) cells that largely lack TRF2 staining. In B, open arrowheads show γ -H2AX-positive (infected, damaged) cells with reduced but detectable TRF1 staining; solid arrowheads show undamaged cells with TRF1 focal staining. C, high level expression of TIN2-13 but not TIN2-14 reduces TRF2 immunostaining. BJ cells were infected with lenti-TIN2-13 or lenti-TIN2-14 and immunostained for TIN2 and TRF2. Nuclei were stained with DAPI (blue), and the images were merged (Merge/DAPI). D, relative effects of TIN2-15C and TIN2-13 on TRF2 immunostaining. HT1080 cells were transiently transfected with vectors (pIRES2-EGFP) that co-express GFP and TIN2-13 or TIN2-15C. The cells were immunostained for TRF2, and the TRF2 and GFP fluorescence images were merged. Nuclei were counterstained with DAPI (blue). Arrowheads, examples of GFP-positive cells and corresponding TRF2 immunostaining. E, TIN2-15C but not TIN2-13 also reduces TRF1 immunostaining. HT1080 cells were transiently transfected with vectors (pIRES2-EGFP) that co-express GFP and TIN2-13 or TIN2-15C. The cells were immunostained for TRF1, and the TRF1 and GFP fluorescence images were merged. Nuclei were counterstained with DAPI (blue). Arrowheads, examples of GFP positive cells and corresponding TRF1 immunostaining. F, TIN2-15 but not TIN2-13 mutants reduce TRF1 and TRF2 protein levels. Lysates from cells expressing TIN2-13, TIN2-15, or TIN2-15C from pIRES2-EGFP were analyzed for TRF1, TRF2, GFP, and tubulin (control) by Western blotting (WB).



or TIN2-15C (Fig. 1C), in yeast and human cells.

First, we used TIN2-13 and TIN2-15 to map the TIN2 regions responsible for TRF2 binding using yeast two-hybrid analysis (38). The results showed the expected TIN2-TRF1 interaction via a central TIN2 domain (15, 16) and confirmed that TIN2 interacts with TRF2 (Fig. 1D). Moreover, the results identified the N-terminal domain of TIN2 (which is dispensable for TRF1 binding (15, 16)) as essential for TRF2 binding (Fig. 1D). Selection against weak contacts using 3-aminotriazole (38) showed that the N terminus (TIN2-15) interacts weakly with TRF2 (Fig. 1D), which we confirmed by *in vitro* translation and immunoprecipitation (not shown). Thus, additional TIN2 domains may influence the strength or stability of the TIN2-TRF2 complex.

To understand the interaction between TIN2 and TRF2 *in vivo*, we analyzed immunoprecipitates from cells expressing FLAG-TIN2 and/or HA-TRF1 (Fig. 2A). When only HA-TRF1 was expressed, anti-HA did not precipitate detectable TRF2 or TIN2, presumably because endogenous TRF1 and TIN2 are complexed with telomeres bound to the nuclear matrix (not shown) (36). However, when both HA-TRF1 and FLAG-TIN2 were expressed, anti-HA precipitated TIN2 and TRF2 (Fig. 2A,

lane 6) with similar efficiencies (compare lane 6 with 9), suggesting that TRF1, TIN2, and TRF2 reside in the same complex *in vivo*. Immunoprecipitations from cells that express Myc-tagged TIN2-15C and HA-TRF1 show that TIN2-15C does not interact with TRF1, despite containing part of the TRF1 interaction domain (Fig. 2B). TIN2-15C, which lacks TRF1 binding, interacted with much less TRF2 than wild-type TIN2 or TIN2-14, both of which retain TRF1 binding and the N terminus (Fig. 2C, compare lanes 5, 6, and 7). This result supports the yeast two-hybrid (Fig. 1D) and *in vitro* immunoprecipitation (not shown) assays, both of which indicated a weak interaction between the N terminus of TIN2 and TRF2, suggesting that the TRF1-binding domain enhances or stabilizes the TIN2-TRF2 interaction. In addition, also confirming the yeast two-hybrid analyses, TIN2-13 binds TRF1 (Fig. 2B) but not TRF2 (Fig. 2C, compare lane 8 with 5 and 7), whereas TIN2-14 binds both TRF1 (15) and TRF2 (Fig. 2C, lane 7). Together these findings indicate that TIN2 binds TRF2 *in vitro* and in cells and that this interaction requires a TIN2 (N-terminal) domain that is distinct from the central TRF1-binding domain.

TIN2 Mutants Induce a Telomeric DNA Damage Response— Telomere dysfunction caused by dominant-negative mutants of

TRF2 induce a damage response characterized by γ -H2AX and 53BP1 nuclear foci (6, 7). If the TIN2-TRF2 complex is crucial for telomere function, its disruption by a mutant TIN2 protein should induce this response. To test this idea, we used lentiviruses to express Myc-tagged TIN2-15C or green fluorescent protein (GFP) (control) in normal human fibroblasts (strain BJ). The viruses use the cytomegalovirus promoter to express the transduced proteins at high levels. GFP fluorescence and anti-Myc immunostaining showed a 70–80% infection efficiency (Fig. 3A). Immunostaining for γ -H2AX showed that 70–80% of cells in TIN2-15C but not GFP expressing cultures had prominent nuclear foci (10–40 foci/nucleus) (Fig. 3B). Moreover, >95% of the TIN2-15C-positive cells were positive for γ -H2AX foci (not shown). Likewise, TIN2-15C (Fig. 3C) but not GFP induced 53BP1 foci, essentially all of which coincided with γ -H2AX foci (Fig. 3D), indicating that these foci were the result of a DNA damage response. Most of the 53BP1/ γ -H2AX foci localized to telomeres, detected by *in situ* hybridization using a protein-nucleic acid probe (Fig. 3E). Thus, overexpression of a TIN2 protein that binds TRF2 but not TRF1 (TIN2-15C) caused a DNA damage response at telomeres. Likewise, a TIN2 protein that binds TRF1 but not TRF2 (TIN2-13), similarly delivered and expressed, induced damage-responsive foci (Fig. 3F). However, TIN2-14, which retains both TRF1 (15, 16) and TRF2 binding (Fig. 2C), failed to induce a DNA damage response (Fig. 3F). Together, these results indicate that disruption of either the TRF1- or TRF2-binding functions of TIN2 causes telomere uncapping.

TIN2 Mutants Destabilize and/or Disrupt TRF2 and TRF1 at Telomeres—To understand how TIN2 mutants induce a telomeric damage response, we immunostained the 53BP1/ γ -H2AX foci-positive and -negative BJ cells in the lenti-TIN2-15C-infected population for TRF1 and TRF2. Most (>95%) cells with 53BP1 or γ -H2AX foci were devoid of focal TRF2 staining, whereas most cells that lacked 53BP1/ γ -H2AX foci had detectable TRF2 staining (Fig. 4A). TRF1 was also affected by TIN2-15C overexpression, albeit to a lesser extent. Only some γ -H2AX-positive cells showed reduced TRF1 staining (Fig. 4B). Thus, TIN2-15C caused a telomeric damage response and reduced TRF2 and, to a lesser extent, TRF1 localization at telomeres. Likewise, TIN2-13 but not TIN2-14 reduced TRF2 immunostaining (Fig. 4C). Together, these findings indicate that TIN2 is important for telomere capping and that it requires both TRF1 and TRF2 binding to maintain functional telomeres.

Similar results were obtained when we transiently co-expressed Myc-tagged TIN2 mutants and GFP in HT1080 (telomerase-positive human tumor cells) using a plasmid that expressed TIN2 proteins from the cytomegalovirus promoter and GFP from an internal ribosomal entry site (pIRES-EGFP). Most (>90%) GFP-positive cells were positive for anti-Myc staining (not shown). TIN2-15C markedly reduced focal (telomeric) TRF2 staining, whereas TIN2-13 was slightly less active in this regard (Fig. 4D). In addition, TIN2-15 (not shown) and TIN2-15C but not TIN2-13 reduced TRF1 staining, albeit less than they reduced TRF2 staining (Fig. 4E).

Because TRF1 is degraded on dissociation from telomeres (39), we quantified the effects of TIN2 mutants on TRF1 and TRF2 protein levels by Western blotting. TIN2-15 and TIN2-15C markedly reduced the TRF1 and TRF2 protein levels, whereas TIN2-13 had little effect (Fig. 4F). Taken together, these findings indicate that TIN2 is important for the stability and localization of both TRF1 and TRF2 at telomeres.

DISCUSSION

Simple organisms such as yeast have one telomeric DNA-binding protein (Rap1 or Taz1) (9, 23, 24), whereas mammals

have two such proteins (TRF1 and TRF2). TIN2 was first identified as a human telomere-associated protein that interacts with TRF1 and mediates its function in telomere length control (15, 16). Here we show that TIN2 also interacts with TRF2 and mediates its function in telomere end protection. Thus, TIN2 may have evolved to connect the functions of TRF1 and TRF2. This possibility may explain why there are no TIN2 homologues in yeast (*S. cerevisiae*, *S. pombe*), flies (*Drosophila melanogaster*), and nematodes (*Caenorhabditis elegans*).

In vitro immunoprecipitation analysis using purified TRF1, TRF2, and TIN2 proteins showed that TIN2 can interact directly with both TRF1 (15, 16) and TRF2. The majority of complexes formed *in vitro* were either TIN2-TRF1 or TIN2-TRF2. However, the interactions with TRF1 and TRF2 required distinct TIN2 domains. The TIN2-TRF1 interaction requires a central TIN2 domain (15, 16), whereas the TIN2-TRF2 interaction required an N-terminal TIN2 domain. This result then left open the possibility that a TIN2-TRF1-TRF2 complex may form *in vivo*. Indeed, *in vivo* immunoprecipitation experiments using lysates from cells overexpressing TRF1 and TIN2 indicated the presence of TRF1-TIN2-TRF2 complexes. Whatever the nature of the TIN2 complex *in vivo*, TIN2 mutants that affect TRF2 but not TRF1 binding (TIN2-15/C) disrupted telomeres by removing TRF2 and to a lesser extent TRF1, indicating an essential role for TIN2 in maintaining a functional, capped structure.

TIN2-15C interacted weakly with TRF2 in immunoprecipitation and yeast two-hybrid assays, despite strongly destabilizing TRF2 by immunostaining. This finding suggests that the TRF1-binding domain of TIN2 stabilizes the TIN2-TRF2 interaction. Expression of TIN2-15C may destabilize TRF2 at telomeres by directly titrating TRF2, in which case it is possible that only a small amount of TRF2 displacement may be sufficient for telomere dysfunction and subsequent loss and destabilization of the remaining TRF2. Alternatively, TIN2-15C may titrate a recently described TIN2-interacting protein, PTP/PIP1, which interacts with the TIN2 N terminus and may affect the TIN2-TRF2 interaction (9, 40). TIN2 mutants that bind TRF1 but not TRF2 (TIN2-13) also disrupted TRF2 localization at telomeres. However, mutants (TIN2-14) that bind both TRF1 and TRF2 did not disrupt TRF2 localization at telomeres. Thus, both TRF1 and TRF2 binding were important for the telomere capping function of TIN2. Telomere uncapping by TIN2 mutants caused a senescent response in normal human cells and an apoptotic response in human tumor cells.² Taken together with data published previously on the TIN2-TRF1 interaction, our findings suggest that TIN2 can connect the activities of TRF1 and TRF2, stabilizing their levels and localization at telomeres, and modulating their capping function.

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- A—Author names: Please verify surnames and capitalization of author name Sahn-ho (or Sahn-Ho?).
- B—If your summary is long enough to run over into the right-hand column, please cut text.
- C—Ref. 29 was a duplicate of Ref. 9. Therefore, Ref. 41 was moved up to 29 and the appropriate changes were made in text. Please verify.
- D—If 'ATM' is an abbreviation and not a designation, please write out.
- E—Sentence 'both in a telomerase-dependent fashion' was expanded for clarity to 'but both wild type and mutant function in a telomerase-dependent fashion. . . ' Please revise if meaning has been changed.
- F—Please verify change '[in EMS assays] TIN2 is shown to form an unusually large complex. . . [and]. . . These results. . . (plural, rather than 'this result' referring to both complex and probe formation okay?).
- G—The term 'Flag' was changed to 'FLAG' per journal style.
- H—Abbreviations, e.g. 'PBS' must be used 5x in text to be retained. Also, RIPA has been defined in footnote 1. Please verify.
- I—Company Pharmacia updated to Amersham Biosciences okay?
- J—Hyphenated complexes were changed from 'anti-TRF2, -TIN2, or -hRap1' to 'anti-TRF2, anti-TIN2, or anti-hRap1' to avoid confusion. Please confirm 'anti-HA-TRF2 or anti-TRF2' is correct as meant.
- K—Please verify amino acids as, e.g. '*-Trp*' italic.
- L—Per your Aug. 10 email, the term 'myc' was capitalized 'Myc' throughout. Please verify.
- M—Sentence 'TRF1 being in' was changed to 'the presence of TRF1 in. . . ' Please verify or revise if not as meant.
- N—Sentence 'Together these results. . . ' and subsequent sentences were run in to avoid single-sentence paragraphs, per journal style
- O—'CMV promoter' was spelled out twice as 'cytomegalovirus.' Please confirm.

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES

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P—Per your Aug. 10 email, the complex 53BP1/ γ -H2AX was set in this order throughout. Please verify.

Q—If 'HT1080 (telomerase-positive human tumor cells)' is new information, should it be transposed to the first citation of HT1080 under EXPERIMENTAL PROCEDURES?

R—If 'pIRES-EGFP' is an abbreviation and not a designation, please write out.

S—Please verify change from 'senescence (noun). . . and apoptotic (adj) response' to 'senescent (adj). . . and apoptotic (adj) response.' Also, per journal style, unpublished material is set as a footnote. Please verify author names in footnote 2.

T—Ref 33: the journal requires that all authors be cited. Please supply all authors' names for this reference.

U—Fig 1B legend: '6Xhis' was changed to 'His₆' per journal style. Also, please verify His-tagged

V—Lables cited in text, e.g '(Input (10%),' have been edited to match the figure

W—Figs 3 and 4: Please check the colors on the computer screen against what appears in the legend and amend the legend as necessary. Are the color figures acceptable for publication?

X—Fig 3, B and C, legend: Please explain (b) and (c). Either explain in the legend or delete, or if these refer to figure labels, you may return a revised hardcopy with proofs.
